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Delineation of a Membrane-proximal β-rich Domain in the $GABA_A$ Receptor by Progressive Deletions

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The type A gamma-aminobutyric acid ($GABA_A$) receptor plays a major inhibitory role in the central nervous system. Structural elucidation of the $GABA_A$ receptor has been impeded by the large size of the receptor. We present here the delineation of a minimal structural domain as the first step of dissecting the receptor structure. This was achieved through prediction-assisted progressive deletions: the prediction of a candidate structural domain rich in β-strands with no close similarity to known structures was tested by deleting putative secondary structure elements from the ends of the proposed domain, as well as mutations within the terminal secondary structures. Such progressive deletions revealed the limits of an integral domain, spanning Cys180 to Met293 (numbering of human α1 subunit). Below these limits the intact domain structure, as indicated by its circular dichroism, collapses. Based on its putative position, this domain is provisionally designated the membrane-proximal β-rich domain of $GABA_A$ receptor. The inclusion of sequences from the first two out of four previously suggested transmembrane segments and one of the two conserved Cys residues in this domain defines important constraints to the receptor structure.

Keywords: far-UV and near-UV circular dichroism; fold-recognition; ligand-gated channel; protein structure prediction; secondary structure

Type A gamma-aminobutyric acid ($GABA_A$) receptors (Barnard, 1995; Macdonald & Olsen, 1994; Sieghart, 1995; Smith & Olsen, 1995; Stephenson, 1995) belong to the ligand-gated ion channel receptor (LGCR) superfamily, and are the targets for many important neuroactive drugs, such as benzodiazepine tranquilizers and anaesthetics. The lack of a direct tertiary structural information on $GABA_A$ receptors has greatly hindered studies on this clinically important class of receptors.

Difficulties in structural studies of the $GABA_A$ receptor mainly arise from the nature of the receptor being a large polymeric transmembrane protein, which presents formidable problems to current structural biology techniques. A dissecting-rebuilding strategy (Campbell & Downing, 1994), which divides the protein into autonomous folding units (domains) and resolves the structures of individual domains prior to reconstitution of the image of the whole protein, represents a reasonable approach for characterising these receptors. Our recent (Xue *et al*., 1998) success in expressing a 131 residue fragment, Cys166-Leu296, of $GABA_A$ receptor suggests the possibility of isolating a minimal domain for structural resolution by this approach.

The $GABA_A$ receptor extracellular region, because it harbors most of the ligand-binding sites (Smith & Olsen, 1995), is of special pharmaceutical interest. The expressed 131 residue fragment, spanning the C-terminal half of the extracellular region, provides a useful starting point for systematic deletions to probe the limits of any integral structural domain it may contain.

Identification of a candidate domain

To assist experimental design, theoretical predictions were performed in an attempt to suggest

Abbreviations used: $GABA_A$, type A gamma-aminobutyric acid; LGCR, ligand-gated ion channel receptor; 3D, three-dimensional; Gu-HCl, guanidose chloride; MPB, membrane-proximal β-rich.

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testable structural domains within the Cys166-Leu296 fragment. While homology modeling is the most powerful tool for protein structure prediction, there is no protein with significant sequence identity (>25%) with $GABA_A$ receptors in the three-dimensional (3D) structure data base usable as a template for homology modeling. Employment of threading programs such as THREADER (Jones *et al.*, 1992) also did not identify any 3D structure templates with Z-score above confidence thresholds.

Circular dichroism measurements (Xue *et al.*, 1998) have shown that the expressed Cys166-Leu296 fragment forms a largely stable structure containing mainly β-strands. Accordingly, a variety of protein-fold recognition programs were screened to determine which of these algorithms could generate β-rich predictions in the vicinity of this fragment. In this regard, the application of both the TOPITS (Rost, 1995, 1996; Rost *et al.*, 1997), and UCLA-DOE (Fisher & Eisenberg, 1996) programs to the full-length mature $GABA_A$ receptor α1 subunit, as well as overlapping 120 residue fragments, yielded an α-helix at the begining of the receptor sequence followed by an essentially all-β stucture over the N-terminal two-thirds of the protein (Figure 1A and B). However, neither one of these two prediction modes could delineate the precise boundaries of a putative all-β domain.

To gain further insight into the possible domain organization within Cys166-Leu296, PHDsec (Rost, 1996; Rost & Sander, 1993a,b), a neural network-based secondary structure prediction program utilizing evolutionary contents of multiple sequence alignment was employed. In the major secondary structure components predicted by PHDsec (Figure 2), the all-β prediction is especially strong in the stretch Leu182-Thr292. Within this stretch, 71 residues, or 64% of the total 111 residues, are predicted to fall within β-strands. This stretch of the receptor indeed would constitute a particularly interesting candidate domain for at least three reasons. First, some residues suggested to be involved in benzodiazepine modulation of $GABA_A$ receptor function (Pritchett & Seeburg, 1991; Wieland & Luddens, 1994; Buhr *et al.*, 1997) are located in this region. Second, it is flanked by the conserved Cys-Cys loop (Cockcroft *et al.*, 1990) at its N terminus and the first two transmembrane segments (Gorne-Tschelnokow *et al.*, 1994; Xu & Akabas, 1996) at its C terminus, which are themselves important structural elements. Third, this portion of receptor sequence resembles in length some commonly encountered extracellular all-β modules (Bork *et al.*, 1996), e.g. C-type lectin (130 residues), immunoglobulin "superfamily" (100 residues), cytokine receptor N-terminal domain (90 residues), and fibronectin type III (90 residues), thereby suggesting size-consistency with a candidate structural domain.

Prediction-assisted progressive deletions

To test experimentally the existence of the putative domain and to delineate its physical boundaries, progressive deletions were performed from both ends of an initial construct encoding the 131 residue Cys166-Leu296 fragment of human $GABA_A$ receptor α1 subunit which, somewhat longer than the putative domain, is known to form stable secondary structures even at high temperature and high pH (Xue *et al.*, 1998). The deletional mutants generated (Table 1) were expressed in *Escherichia coli* and purified (Figure 3(a)) for

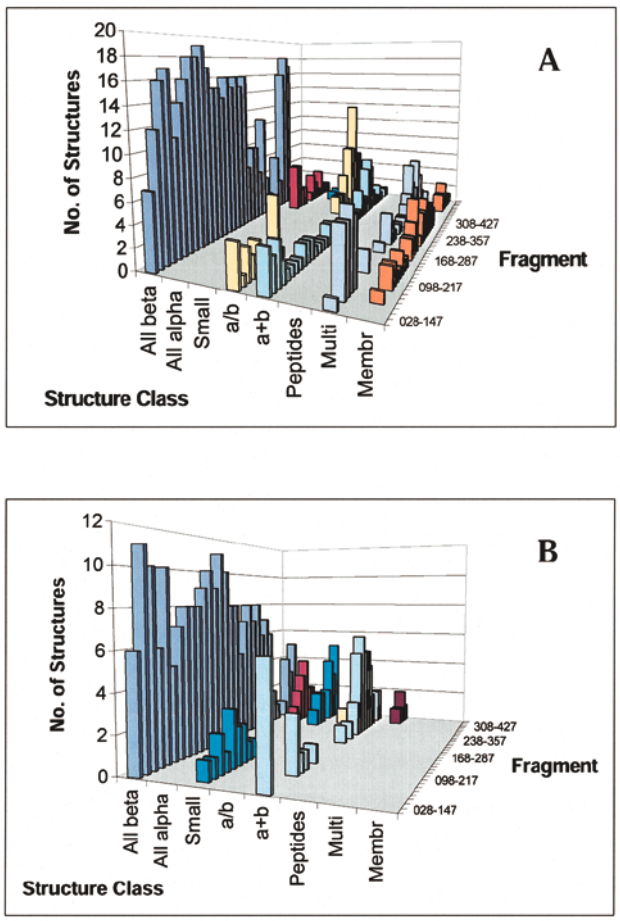

Figure 1. Fold recognition using sequence-derived predictions. The top 20 known protein 3D structures identified by (A) TOPITS (Rost *et al.*, 1997) or top 15 by (B) UCLA-DOE (Fisher & Eisenberg, 1996) in terms of similarity to overlapping 120 residue fragments of $GABA_A$ receptor α1 subunit, with a scanning interval of ten residues, are grouped in structure classes according to SCOP classification (Hubbard *et al.*, 1997). Identified protein classes include: All beta, all-β; All alpha, all-α; Small, small protein (usually dominated by metal ligand, heme, and/or disulfide bridges); a/b, α and β (mainly parallel β sheets; β-α-β units); a + b, α and β (mainly antiparallel β sheets; segregated α and β regions); Peptides, peptides and fragments; Multi, Multi-domain (consisting of more than one domain of different classes); and Membr, membrane and cell surface proteins and peptides.

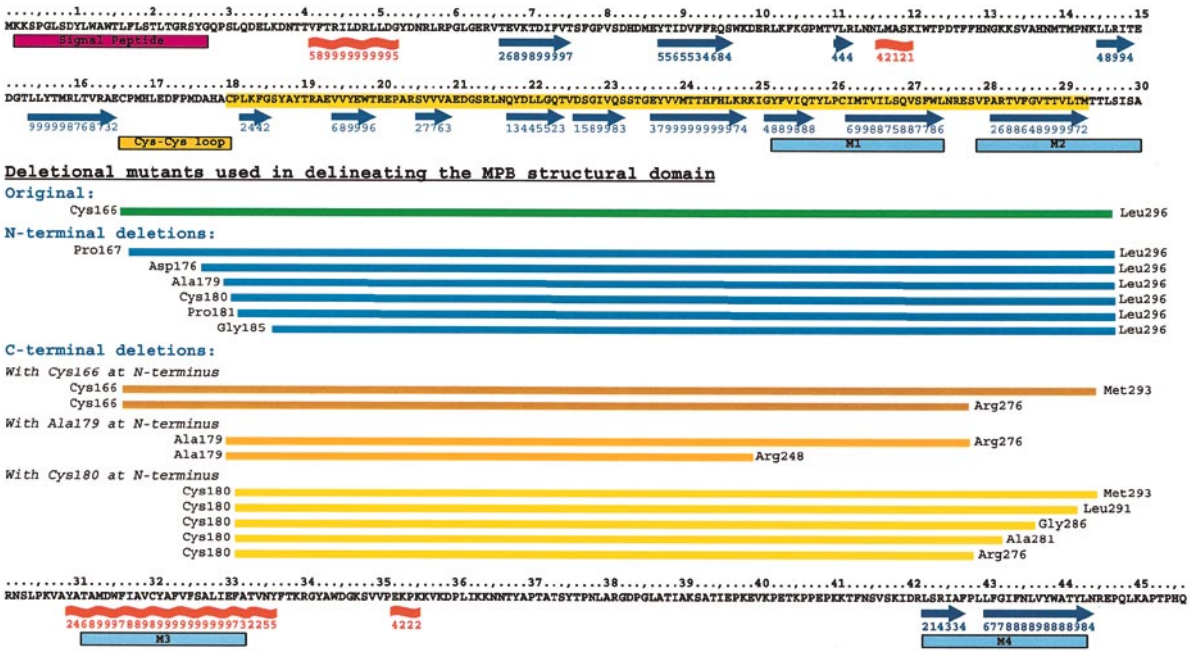

Figure 2. The minimal MPB structural domain in $GABA_A$ receptor α1 subunit. The yellow highlighted sequence indicates the span of the minimal structural domain. Secondary structures predicted by PHDsec (Rost, 1996) and other putative structural features (Olsen & Tobin, 1990; Schofield *et al.*, 1987) are labeled for the human $GABA_A$ receptor α1 subunit sequence. The numbers under the red curvy lines (α-helix) and blue arrows (β-strand) indicate the strength of the predictions. The greater the number, the stronger the prediction, with 9 as the strongest. M1, M2, M3 and M4, the first, second, third and fourth hypothetical transmembrane segments of Schofield *et al.* (1987). The deletional mutants are represented by color bars aligning with corresponding amino acid sequences.

subsequent investigation by means of far-UV CD spectroscopy as described (Xue *et al.*, 1998). Some of the mutant fragments could be expressed at higher levels than others (Table 1). The fragments difficult to express also tended to be low in solubility.

Near-UV circular dichroism

The initial fragment Cys166-Leu296 was characterized by near-UV CD spectral analysis to detect tertiary packing around the aromatic chromophores. At 0 M guanidine chloride (Gu-HCl), Cys166-Leu296 showed distinct signals from 260 to 295 nm (continuous line in Figure 4), with the major band close to 280 nm displaying pronounced negative ellipticities. In contrast, no apparent CD signal was observed in the near-UV range in the presence of 6 M Gu-HCl (broken line in Figure 4). These results demonstrate that specific side-chain packing exists in fragment Cys166-Leu296, which is disrupted in the presence of denaturant. It is therefore evident that fragment Cys166-Leu296 contains not only stable secondary structures, but also defined tertiary structures. This fragment thus provides an appropriate starting point for deletional analysis.

Deletions of putative secondary structures

A number of deletions were performed on predicted secondary structural elements within fragment Cys166-Leu296 to determine whether these elements were essential for the physical integrity of the postulated domain. Deletion of the Cys-Cys loop spanning Cys166 to Cys180, and the predicted short β-strand formed by Leu182-Phe184 immediately behind the Cys-Cys loop, caused gross changes in the CD spectrum (Figure 3(b)), indicating extensive disruption of secondary structures. Further N-terminal deletions (Table 1) either produced evident changes in CD spectrum as in Ala192-Leu296, or caused severe host growth inhibition as in Pro202-Leu296 and Asn216-Leu296. Extensive CD spectral changes were also observed in mutants with C-terminal deletions: deletion of the last putative β-strand, leaving Cys166-Arg276, Ala179-Arg276 and Cys180-Arg276 (Figure 3(c)), or the last three β-strands, leaving Ala179-Arg248 (Figure 3(c)), all brought about pronounced changes in CD spectrum.

Mutations within the terminal secondary structures

To further pin-point the boundaries of the candidate domain, smaller deletions were performed on the Cys-Cys loop and the last β-strand. Starting from Cys166, five N-terminal deletional mutants were constructed, with a deletion of 1, 10, 13, 14 or 15 residues, respectively. Deletion of 1, 10, 13 or 14 residues did not cause any obvious structural change judging from their CD spectra. However, further deletion of Cys180 gave rise to an observa-

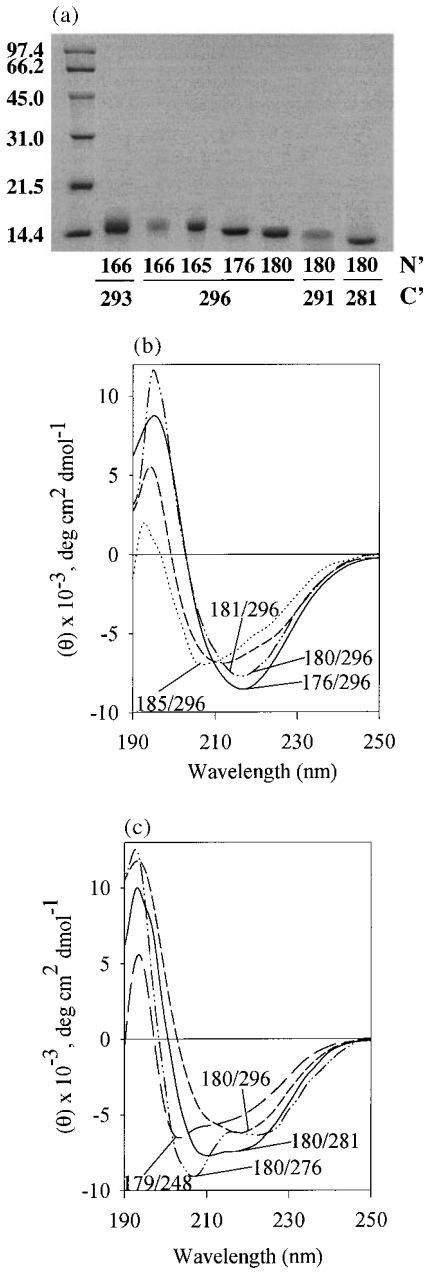

Figure 3. Characterization of recombinant $GABA_A$ receptor fragments. (a) Gel eletrophoresis of representative fragments was carried out in SDS-15% PAGE, with 10% β-mercaptoethanol added to the loading buffer. Proteins in the gels were visualized by Coomassie brilliant blue R250 staining. The left lane is the low-range protein molecular mass standard (from BioRad), which is followed by, from the left: fragments Cys166-Met293, Cys166-Leu296, Pro165-Leu296, Asp176-Leu296, Cys180-Leu296, Cys180-Leu291 and Cys180-Ala281. CD spectra of representative N-terminal (b) and C-terminal (c) deletants were recorded on a JASCO J720 spectropolarimeter at 22 °C. Purified recombinant polypeptides were extensively dialyzed with 10 mM Tris-HCl at pH 8.5. The solutions used for CD measurements were prepared by proper dilution of a stock containing about 8 μM protein. Spectra obtained from five times concentrated samples were essentially superimposable to the ones presented here. Protein concentrations were calculated based on extinction coefficients (Gill & von Hippel, 1989). Each spectrum was an accumulation of 20 scans.

ble change in the CD spectrum of fragment Pro181-Leu296 (Figure 3(b)).

The requirement of Cys180 for the integrity of the domain was confirmed by the substitution of Cys180 with Ala in the fragment Ala179-Leu296. This mutation disrupted the domain structure and caused severe precipitation of the resulting fragment. In contrast, the deletion of Cys166 to generate fragment Pro167-Leu296 brought about little change in the CD spectrum, showing that Cys166 is not an essential stabilizing factor for the secondary structures of the domain.

From the C-terminal side, progressive deletions of 3, 4, 5, 10 or 15 residues were made. The removal of three residues incurred no detectable CD change in the fragments Cys166-Met293 and Cys180-Met293. The other fragments that carry larger C-terminal deletion, namely Cys180-Leu291, Cys180-Gly286 and Cys180-Ala281, all displayed varying degrees of CD spectral changes (Figure 3(c)). Since the effects of the C-terminal deletions were similar for fragments having Cys166, Ala179 or Cys180 at their N termini, clearly the C-terminal effects on secondary structure are not dependent on residues 166-179 at the other end of the polypeptide.

Structural disruptions monitored by CD spectroscopy

Systematic changes in CD spectra also became observable with deletions proximal to Cys180 at the N terminus, or Met293 at the C terminus, the most prominent change being a blue-shift of λ_{max} (Figure 3(b) and (c)) relative to the original λ_{max} of fragment Cys166-Leu296 at 217 nm. In general, the larger the deletion, the shorter was the λ_{max}. The λ_{max} of, for example, fragment Ala179-Arg248 occurred at 203 nm (Figure 3(c)). This blue-shift caused by the deletions could best be explained in terms of secondary structural disruptions giving rise to a larger proportion of random coil-like structures, which are known to exhibit a characteristic negative band around 200 nm.

Moreover, all fragments below the N and C-terminal limits of Cys180-Met293 exhibited some degree of structural instability. Their CD spectra readily varied with time, temperature increase, pH increase and buffer composition; their λ_{max} also shifted to shorter wavelengths upon prolonged storage. In contrast, the CD spectra displayed by longer fragments, such as Cys166-Leu296, were constant under various conditions (Xue *et al.*, 1998), and the λ_{max} of their CD spectra did not shift toward shorter wavelengths after prolonged storage for one week at 4 °C.

Implications

The nature of the domain

Starting with secondary structure predictions, the boundaries of a structural domain within

Table 1. Deletional mutants used in delineating the MPB structural domain

Fragment	Amino acid	M_r (kDa)	Growth	Expression	Solubility	λ_{max} (nm)
A. *Original*						
Cys166-Leu296	131	14.83	+++	+++	+++	217
B. *N-terminal deletions*						
Pro167-Leu296	130	14.73	+++	+++	+++	217
Asp176-Leu296	121	13.63	+++	++	++	217
Ala179-Leu296	118	13.31	++	++	+	217
Cys180-Leu296	117	13.24	++	++	++	217
Pro181-Leu296	116	13.13	+	++	±	211
Gly185-Leu296	112	12.65	++	++	+	207
C. *C-terminal deletions*						
With Cys166 at N terminus						
Cys166-Met293	128	14.52	+++	+++	+++	217
Cys166-Arg276	111	12.73	±	±	±	207
With Ala179 at N terminus						
Ala179-Arg276	98	11.20	±	±	±	207
Ala179-Arg248	70	7.88	++	++	+	203
With Cys180 at N terminus						
Cys180-Met293	114	12.92	+++	+++	+++	217
Cys180-Leu291	112	12.69	+++	++	+	211
Cys180-Gly286	107	12.17	+++	++	±	208
Cys180-Ala281	102	11.61	+++	++	+	209
Cys180-Arg276	97	11.13	±	±	±	207

Cys166-Leu296 have been delineated experimentally by assessing the effects of progressive deletions on secondary structures reflected in far-UV CD spectra. Given the essential requirement of Cys180, and the instability attendant on the deletion of Met293, the minimal domain consistent with physical stability is Cys180-Met293. The essential role of Cys180 within the Cys-Cys loop for the domain structure was supported by not only the deletions, but also the substitutions at this residue. The carboxyl terminus of the domain at Met293 is located at the end of a predicted β-strand within the confines of the previously suggested second-transmembrane segment (Xu & Akabas, 1996).

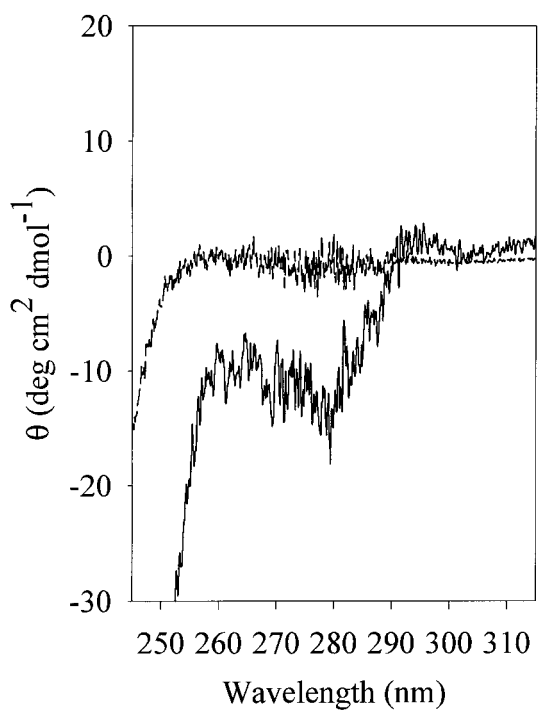

Figure 4. The near-UV CD spectra of fragment Cys166-Leu296 in the absence (broken line) and presence (continuous line) of 6 M Gu-HCl. The protein sample of 100 μM was in 10 mM Tris-HCl, pH 8.5. and equilibrated for measurement at room temperature.

The requirement of sequences from the first two putative transmembrane segments, namely Tyr252-Met293, for domain integrity was clearly indicated by the disruptive effects upon removal of these sequences from the C terminus of the domain (Figure 3(c)).This suggests that these sequences are either part of the domain *per se*, or so closely supportive of the domain as to become indispensable for domain stability. That the domain ends with a cluster of hydrophobic residues in the region of Tyr252-Met293 points to the possibility of the identified minimal domain being anchored into or juxtaposed to the cell membrane. The identified Cys180-Met293 domain, therefore, may be tentatively designated as the membrane-proximal β-rich, or MPB, domain of $GABA_A$ receptor.

The secondary structure composition of the MPB domain was determined by CD spectroscopy to be β-strand dominated. In fact, the CD spectra of all deletional mutants that retained stable structures under the tested conditions remained β-strand-rich in character, with β-strand composition estimated as described by Sreerama & Woody (1994) to be in the neighborhood of 50 %. However, the fold-type of the domain could not be inferred from the present study. Given the low Z-scores in protein-fold predictions by both TOPITS and UCLA-DOE, the identified domain may exhibit considerable deviations from known folds, or even form a new type of β-rich fold.

Structural models of the receptor

The current understanding of the molecular structure of $GABA_A$ receptor has been heavily based on analogy with the prototype of LGCR, the nicotinic acetylcholine receptor (Unwin, 1996). Thus, each subunit of the quasi-symmetric $GABA_A$ receptor pentamer (Nayeem *et al*., 1994) is postulated to contain a putative extracellular region followed by four transmembrane segments, with a cytoplasmic region interposed between the third and fourth membrane-spanning regions (Olsen & Tobin, 1990; Schofield *et al*., 1987). In this current model, the extracellular region of $GABA_A$ receptor is usually represented as a loop-like structure with putative *N*-glycosylation sites, and a disulfide bond formed between the two conserved Cys residues.

More recently, Gready *et al*. (1997) suggested a two-domain model for the extracellular region of LGCR superfamily members on the basis of their computations, using as an example the Gly receptor sequence. Application of this Gready model to human $GABA_A$ receptor α1 subunit would suggest at the N terminus an elaborated SH2 domain consisting of both α-helices and β-strands, followed by, sequentially, first a Cys166-Cys180 disulfide bridge, secondly a long random coil, and finally a cut-down SH3 domain that is β-rich. While the modeled SH3 domain and the MPB domain delineated here by progressive deletions are both β-rich in character, the cut-down SH3 domain modeled by Gready *et al*. (1997) consists of only about 43 residues. In contrast, the MPB domain is 114 residues long. The precise relationship between the SH3 and MPB domains, therefore, remains to be elucidated.

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